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14. ABSTRACT
In the proposal, we hypothesized that HDAC inhibitors will sensitize the effects of ionizing radiation (IR) through inhibition of pro-survival events with simultaneous up-regulation of pro-apoptotic events. The following three specific aims were proposed: Aim 1. To determine the combined effects of HDAC inhibitors and ionizing radiation on prostate cancer cell lines (PC-3, LN-3, LnCAP, DU-145 and 22Rv1). Aim 2. To understand the signaling pathways induced by combined exposures to HDAC inhibitors and ionizing radiation in both androgen dependent and independent prostate cancer cell lines. Aim 3. To determine the combined effects of HDAC inhibitors plus ionizing radiation on the regression of (i) prostate cancer xenografts (PC-3) in nude mice and (ii) in-situ prostate tumor in TRAMP mice. The following tasks were achieved: Aim 1: The radiosensitizing effects of VAD-18 and VAD-20 were studied in PC-3 cells. Further, due to change in the inhibitors for the remaining part of the grant, effects of SAHA and (S)-HDAC-42 were investigated in PC-3, LN-3 and DU-145 cells. (S)-HDAC-42 and SAHA could sensitize PC-3 and DU-145 cells to radiation. Aim 2: Effects of VAD-18 and VAD-20 were mediated through cell cycle arrest, down-regulation of anti-apoptotic proteins, upregulation of pro-apoptotic proteins and abrogation of radiation-induced nuclear translocation of p65, thereby, enhancing cell death. Further, a novel transcription factor, USF-1 was identified which may be responsible for the radiosensitizing effects of these inhibitors. Aim 3: The breeding of TRAMP mice is in progress. We are currently increasing the population of true TRAMP mice for further experiments. Ultrasound Imaging protocol will be used for assessing in situ regression of tumors.

15. SUBJECT TERMS
Histone deacetylase, radiosensitization, apoptosis, NF-kappaB, Prostate cancer, TRAMP, USF-1

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I INTRODUCTION

Transcriptional control of gene expression is intimately linked to the post-translational modification of chromatin by acetylation, methylation or phosphorylation. Further, acetylation status of the chromatin has been shown to have fundamental importance in the initiation or progression of cancer. Acetylation status of the chromatin, modulated by histone acetylases (HATs) and histone deacetylases (HDACs), is responsible for chromatin remodeling that is required for gene expression (1-3). HATs acetylate histones at the lysine residues thus neutralizing the charge (2, 4). The resulting relaxation of the nucleosomal core particle leads to transcriptional activation (5, 6). HDACs on the other hand, remove acetyl groups from acetylated histones leading to chromatin compaction and transcriptional repression (5-7). HDACs have been shown to target not only histones but several other transcription factors like RB, p53, NFκB, ATM and MEF2 for deacetylation (7).

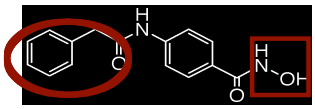
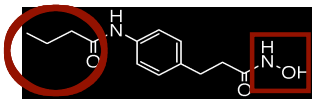
Since, aberrant activity of HDACs leads to the transcriptional repression of tumor suppressor genes contributing to tumor formation (7), targeting of HDACs with inhibitors would not only disrupt normal transcriptional regulation of specific genes through the relaxation of chromatin conformation but also can be used as a cancer therapy approach. Indeed, several types of HDAC inhibitors have been shown to have anti-tumor activities in both tumor cells and xenografted models (8-13). Due to the success of HDAC inhibitors in preclinical studies, Phase I and II clinical trials of several different inhibitors have been initiated (14-17). In addition to anti-tumor activity, HDAC inhibitors have shown several biological effects-morphological changes (18), transcriptional changes (19, 20), cell differentiation (21), cell cycle arrest (22, 23), antiangiogenesis (24) and apoptosis (23, 25-27) (reviewed in (6)).

Several distinct classes of HDAC inhibitors have been reported including short chain fatty acids (28, 29), benzamide derivatives (30), trichostatin and analogues (31, 32), hybrid polar compounds (33), cyclic tetrapeptides (34, 35) and the depsipeptide (36). Among these, short chain fatty acids are least potent and their efficacy has been limited by low antiproliferative activity, rapid metabolism and non-specific mode of action (37). Based on the X-ray crystallographic structure of HDAC enzyme, Zn^{2+}-chelating, motif-tethered, short chain fatty acids were developed as novel class of HDAC inhibitors (37). Two of these inhibitors (VAD-18 and VAD-20) having phenylacetic acid and butyric acid respectively as the lead compound (Fig. 1) have been used in the present study. Since, these inhibitors have aromatic chain (rather than aliphatic chain present in most other inhibitors) as the linker between the lead compound and Zn^{2+} chelating hydroxamic acid, there is more strong interaction between the hydrophobic pocket of the active site of the enzyme and the inhibitor thus increasing the potency of inhibition (37). Indeed, these inhibitors alone showed upregulation of $p21^{WAF/CIP1}$ expression and hyper-acetylation of histones H-3 and H-4 in DU-145 cells at concentrations significantly lower than the parent molecule phenylbutyrate (37).

Lu *et al* (38) further embarked on the structure-based optimization of these inhibitors by using the framework generated by the crystal structure of histone deacetylase-like protein (HDLP)-TSA complexes. Based on the hypothesis that the hydrophobic residues flanking the cap group-binding motif could be exploited

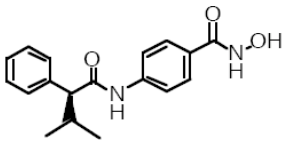
for lead optimization, they generated (*S*)-**11** ((S)-HDAC-42), an optically active α-branched phenylbutyryl derivative, with IC_{50} of 16 nM in HDAC inhibition (Fig. 2). (S)-HDAC-42 also showed hyper-acetylation of H3 in PC-3 cells (39). Statement of work for the proposal was therefore modified and VADs were replaced with (S)-HDAC-42, a second generation inhibitor. The effects of this inhibitor in combination with radiation will be compared with SAHA.

Fig. 1 Structures of VAD-18 and VAD-20.

HDAC inhibitors	Structures	Lead compound
VAD-18		Phenylacetic acid
VAD-20		Butyric acid

Circle shows capping groups and the square shows Zn^{2+} chelating groups

Fig. 2 Structure of (S)-HDAC-42.



(*S*)-(+)-*N*-hydroxy-4-(3-methyl-2-phenyl-butyrylamino)benzamide
HDAC inhibition: IC_{50}, 16 nM
DU-145 growth inhibition, IC_{50}, 110 nM

One of the most important problems in prostate cancer research is the need to identify a treatment for radiation resistant prostate cancer. Radiation resistance in prostate cancer may be implicated to induction of pro-survival factors by radiation itself. These radiation-induced pro-survival factors may provide anti-apoptotic signal to evade from cell killing effects of radiation. It may be possible to inhibit the functions of radiation-induced pro-survival factors and enhance radiation-induced apoptosis by the use of several drugs. HDAC inhibitors can be used in combination with radiation to augment clinical efficacy and/or to reduce toxicity. The HDAC inhibitors- phenyl butyrate (40), sodium butyrate (41), trichostatin A (42-44), SAHA (44), M344 (44), depsipeptide (44) and a benzamide MS-275 (45) have shown tumor cell radio-sensitivity in various cancer cell lines. However, since sodium butyrate and trichostatin A have limitations as mentioned above and in addition the mechanisms of radio-sensitization by these inhibitors have not been studied, the present study was warranted to investigate the effects of novel HDAC inhibitors, VAD-18, VAD-20 and (S)-HDAC-42 in various prostate cancer cell lines in combination with radiation and to compare the effects with SAHA as well as to understand the mechanisms behind radio-sensitization.

II BODY

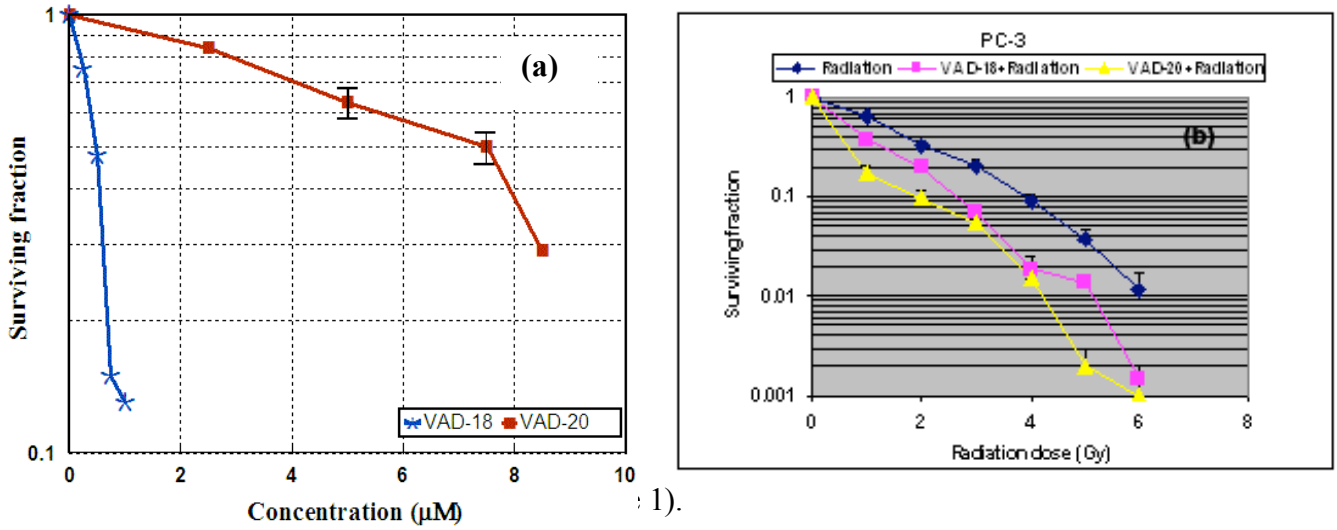
Task 1. To determine the combined effects of HDAC inhibitors and ionizing radiation on prostate cancer cell lines (PC-3, LN-3, LnCAP, DU-145 and 22Rv1). This will be assessed for clonogenic inhibition and apoptosis.

a. Perform colony forming assays to estimate the reproductive death induced in the presence of HDAC inhibitors and radiation.

b. Perform TUNEL assay and flow cytometric measurements of DNA content for studying the HDAC inhibitors-induced apoptosis in the presence or absence of radiation.

HDAC inhibitors inhibit HDAC enzymes leading to hyper-acetylation of the chromatin and thus affecting the transcription of several genes. Inhibition of the radiation-induced pro-survival genes by these inhibitors and simultaneous induction of pro-apoptotic genes irrespective of androgen functional status will dominantly lead to enhanced cell death. Thus, we studied the cell death in presence of these inhibitors with and without irradiation in these cells. Clonogenic survival and apoptosis of these cell lines in the presence of HDAC inhibitors alone or in combination with ionizing radiation treatment was determined by colony forming assay and flow cytometry (sub G_0/G_1 population).

Task 1 a: PC-3 cells were treated with various concentrations of VAD-18 and VAD-20 to find the IC_{50} concentration for each drug. The decrease in surviving fraction with increasing concentration was observed with both the drugs (Fig. 3 a). However, VAD-18 was more cytotoxic than VAD-20 with the corresponding IC_{50} concentrations of 0.5 μM and 7.5 μM. The effects of VAD-18 (0.5 μM) and VAD-20 (7.5 μM) in combination with radiation (1-6 Gy) on survival of PC-3 cells are presented in Fig. 3b. Colony forming assays showed significant radio-sensitizing effects with both the inhibitors at IC_{50} concentrations in PC-3 cells (SF_2=0.2±0.013; D_0=145 cGy for VAD-18 + IR and SF_2=0.1±0.02; D_0=122 cGy for VAD-20 + IR) compared to



: 1).

Fig. 3 Effects of **(a)** VAD-18 and VAD-20 alone and **(b)** VAD-18 (0.5 μM) and VAD-20 (7.5 μM) with radiation (1-6 Gy) on surviving fraction of PC-3 cells studied by colony forming assay.

IC_{50} concentrations of (S)-HDAC-42 and SAHA were calculated using clonogenic survival assay in PC-3, LN-3 and DU-145 cells. LN-3 cells (p53 wild type; partially androgen-dependent) were slightly more sensitive to both (S)-HDAC-42 and SAHA compared to DU-145 (p53 mutated; androgen-independent) and PC-3 (p53 null; androgen-independent) cells (Fig. 4 a and b; Table 2). Radiosensitizing effects of (S)-HDAC-42

Table 1: Inactivation estimates of various HDAC inhibitors in PC-3 cells with or without radiation.

Treatment	Inactivation estimates			Radiation enhancement ratios
	IC_{50}	SF_2	D_0 (cGy)	
Radiation (IR)	-	0.33±0.031	148.8	-
VAD-18	0.5 μM	-	-	-
VAD-20	7.5 μM	-	-	-
VAD-18+IR	-	0.2±0.013	145	1.3
VAD-20+IR	-	0.1±0.02	122	2.3
(S)-HDAC-42	20 nM	-	-	-
(S)-HDAC-42+IR	-	0.0585±0.0015	42	4.6
SAHA	0.2 μM	-	-	-
SAHA+IR	-	0.118±0.025	79	2.3

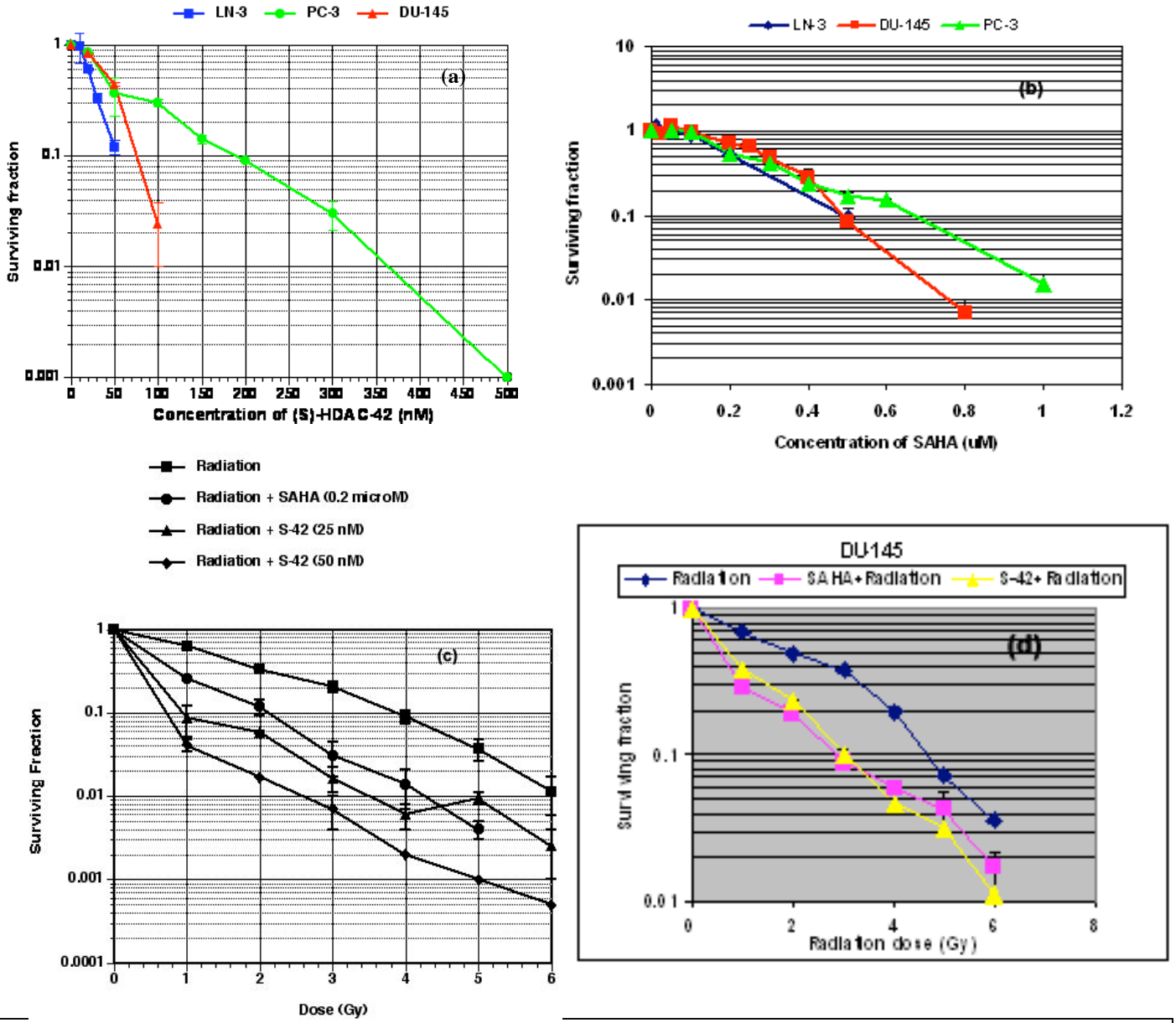


Fig. 4 Effects of **(a)** (S)-HDAC-42; **(b)** SAHA alone in LN-3, PC-3 and DU-145 cells; **(c)** (S)-HDAC-42 (25 & 50 nM) and SAHA (0.2 μM) with radiation (1-6 Gy) on surviving fraction of PC-3 cells and **(d)** (S)-HDAC-42 (25 nM) and SAHA (0.25 μM) with radiation (1-6 Gy) on surviving fraction of DU-145 cells studied by colony forming assay.

and SAHA were investigated in PC-3 and DU145 cells using colony forming assays (Fig. 4 c and d). (S)-HDAC-42 was able to enhance the radiation effects much more effectively than any of the other inhibitors: VAD-18, VAD-20 or SAHA in PC-3 cells (Figs. 3 b, 4 c; Table 1). In DU-145 cells, however, slightly better radiosensitization effects were obtained with SAHA than (S)-HDAC-42 (Fig. 4 d; Table 3).

Table 2: IC_{50} concentrations of (S)-HDAC-42 and SAHA in PC-3, LN-3 and DU-145 cells determined from clonogenic survival assays.

Cell line	IC_{50}	
	(S)-HDAC-42	**SAHA**
PC-3	20 nM	0.2 μM
LN-3	20 nM	<0.2 μM
DU-145	25 nM	0.25 μM

Table 3: Inactivation estimates of various HDAC inhibitors in DU-145 cells with or without radiation.

Treatment	Inactivation estimates			Radiation enhancement ratios
	IC_{50}	SF_2	D_0 (cGy)	
Radiation (IR)	-	0.5±0.0115	216	-
(S)-HDAC-42	25 nM	-	-	-
(S)-HDAC-42+IR	-	0.24±0.0045	121	1.9
SAHA	0.25 μM	-	-	-
SAHA+IR	-	0.19±0.0045	99.5	2.4

Task 1 b: Cell cycle analysis: Since, HDAC inhibitors are known to influence the cell cycle distribution, effects of these inhibitors on cell cycle distribution were analyzed by flow cytometry in PC-3 cells (Fig. 5). A significant transient block in G_2/M phase of the cell cycle was observed with either 2 Gy of radiation or drugs alone up to 12 h post-treatment. Exposure to VAD-18 resulted in an additional delay following irradiation, which was significant, while VAD-20 had a lesser effect. An increase in percent S-phase was also observed following treatment of cells with either of the drugs till 24 h. However, at later time points (after 24-72 h), the block was released. To investigate whether sustained arrest in G_2/M phase could be observed, cell cycle distribution studies were performed with higher concentrations of VAD-18 (0.5 to 1 μM) and VAD-20 (7.5 to 15 μM). However, similar results were obtained as with IC_{50} concentrations (data not shown). Since, the hypodiploid peak was apparent only at 36 h and later, it appears that only delayed apoptosis was enhanced in combined treatment as cells blocked in G_2/M harbor more damage, which could lead either into apoptosis at a later time or manifest enhanced cytogenetic damage after the release of G_2/M block and lead to secondary apoptosis. Indeed, analysis of cells by fluorescence microscopy showed enhanced cytogenetic damage with radiation and the combination (data not shown).

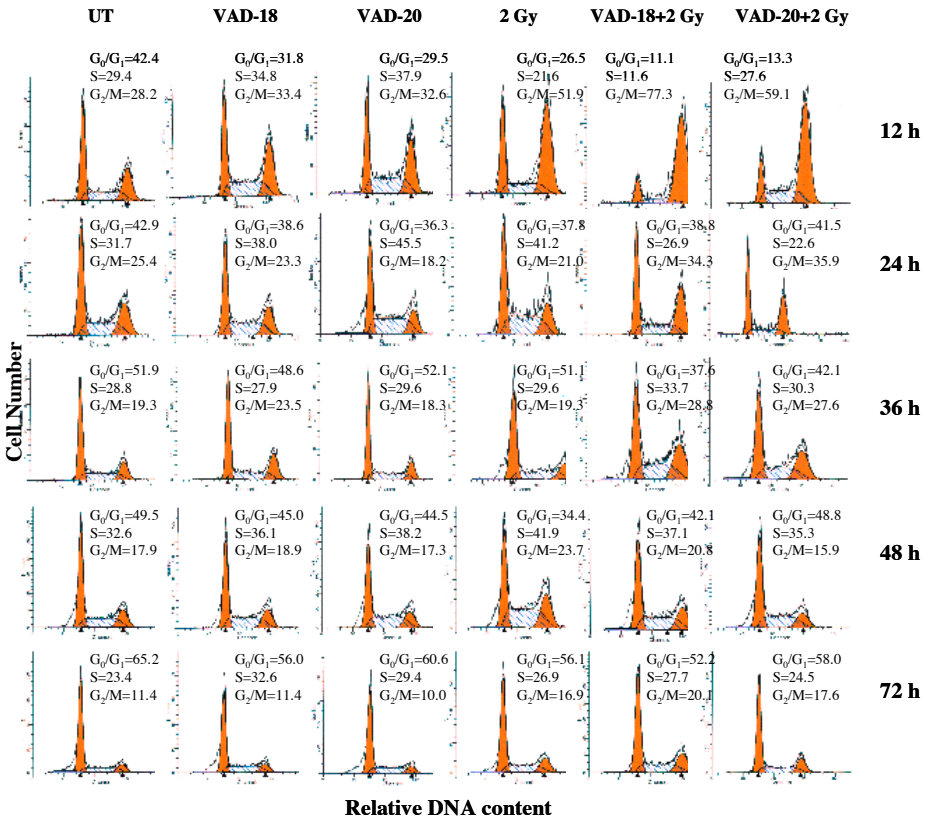


Fig 5. Effects of VAD-18 (0.5 μM) and VAD-20 (7.5 μM) with radiation (2 Gy) on cell cycle distribution analyzed by flow cytometry following staining of cells with propidium iodide in PC-3 cells.

Task 2. To understand the signaling pathways induced by combined exposures to HDAC inhibitors and ionizing radiation in both androgen dependent and independent prostate cancer cell lines. In particular, effects on pro-survival events will be assessed by analyzing androgen receptor-mediated and NFkappaB-mediated signaling pathways. Further, pro-apoptotic effectors such as Bax induction, cytochrome C release and caspase activity will be assessed in response to this combined treatment.

a. Perform Western blot analyses for quantifying the levels of NFkappaB sub-units (p65 and p50), IkappaB-alpha, p-IkappaB-alpha, Bax, Bcl-2, Bcl-XL, cytochrome C, p21, AR protein, PI-3 kinase, AKT, phospho-AKT, PTEN following the combined treatment either in whole cell lysates or cytosolic and nuclear extracts.
b. Perform electrophoretic mobility shift assays (EMSA) and reporter assays (using 3x kappaB-Luc and AR-Luc) for assessing the transactivation activity of NFkappaB.
c. Perform immunofluorescence staining for analyzing cytochrome C and Bax release and p65 localization following the combined treatment.
d. Measure caspase activity by fluorometric method.

Task 2 a: Western Blot analysis: HDAC inhibitors inhibit HDAC enzymes leading to hyper-acetylation of the chromatin and thus affecting the transcription of several genes. Inhibition of the radiation-induced pro-survival genes by these inhibitors and simultaneous induction of pro-apoptotic genes irrespective of androgen functional

status will dominantly lead to enhanced cell death. Thus, we studied the expression and function of different proteins responsible for either survival or death of cells in presence of these inhibitors with and without radiation using Western blot analysis in PC-3 cells.

Expressions of p65 (one of the components of NFκB, a survival protein) and pro-apoptotic protein (Bax) and anti-apoptotic proteins (Bcl_{XL} and Bcl_2) following treatment with HDAC inhibitors and radiation (2 Gy) were analyzed by Western blot analysis in either cytosolic or nuclear fractions. While, with all the treatments, p65 protein was present in the nuclear extract 1 h post-treatment, its expression decreased at 3 h and again an increase was observed from 6 h post-treatment except in V20 and V20+IR group (Fig. 6 a). This second appearance of p65 in the nucleus was more with IR alone. These time dependent changes in p65 localization and therefore its activity were further confirmed with immunofluorescence and gel shift assays and are described below. Since, the combination treatment resulted in less total p65 in the nucleus compared to 2 Gy treatment, this implies that the inhibitors may sensitize the cells to IR by reducing the levels of IR-induced p65 translocation in the nucleus (Fig. 6 a).

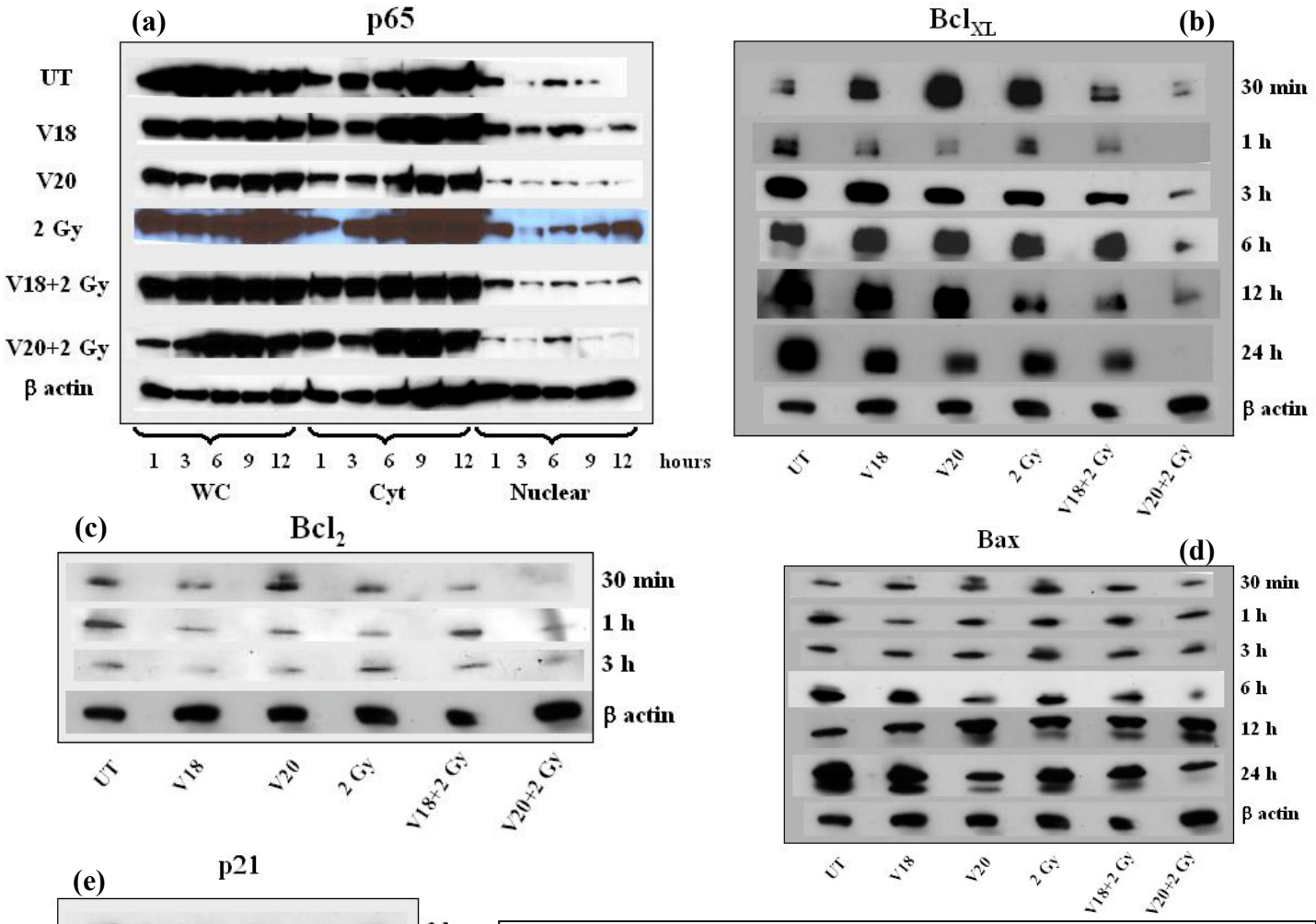


Fig. 6 Effects of VAD-18 (0.5 μM) and VAD-20 (7.5 μM) with radiation (2 Gy) on levels of **(a)** p65 protein in whole cell (WC), cytosolic (Cyt) and nuclear extracts, **(b)** Bcl_{XL}, **(c)** Bcl_2, **(d)** Bax and **(e)** p21 in whole cell extracts of PC-3 cells analyzed by Western blotting. SDS-PAGE was performed with equal loading of the proteins followed by transfer of proteins to PVDF membranes. Bands were visualized by ECL. Representative β actin levels are shown as internal control.

Consistent with the presence of p65 in the nucleus, levels of anti-apoptotic protein, Bcl_{XL}, a target of p65 increased from 30 min to 6 h post-treatment in all the groups except V20+2Gy, where the levels were consistently reduced from 30 min to 24 h post-treatment (Fig. 6 b). Expression of Bcl_{XL} was however reduced in all the groups compared to untreated group at 12 and 24 h post treatment time (Fig. 6 b). Levels of Bcl_2, another anti-apoptotic member of Bcl_2 family of proteins, remained almost unchanged except for V20+IR group where the levels were significantly reduced compared to untreated group (Fig. 6 c). Time-dependent changes in the expression of pro-apoptotic Bax protein are shown in Fig. 6 d. Significant increase in the levels was observed only after 12 h of treatment in all the groups except V20+IR. Since, the ratio of pro- to anti-apoptotic proteins determines the response, the enhanced cell death observed in combination treatment groups especially with combination of VAD-20 and radiation could be due to an increase in this ratio.

Since, most of the HDAC inhibitors have been shown to increase the levels of p21 protein (responsible for blocking the cells in G_1 phase of the cell cycle), expression of p21 protein was investigated in PC-3 cells (Fig. 6 e). Consistent with the cell cycle distribution data, significant increase in the levels of p21 was observed only after 24 h of treatment. This delayed induction of p21 may also be responsible for G_2/M block of the cells.

Task 2 b: Electrophoretic mobility gel shift assay: To confirm that p65 is able to bind to its target after localization in to the nucleus, electrophoretic mobility gel shift assay (EMSA) was performed in PC-3 cells. An increase in p65 binding was observed with either the drugs alone or radiation alone at 1 h but the binding was reduced at 3 h followed by an increase again from 6 h post-treatment (Fig. 7). VAD-20 in combination with 2

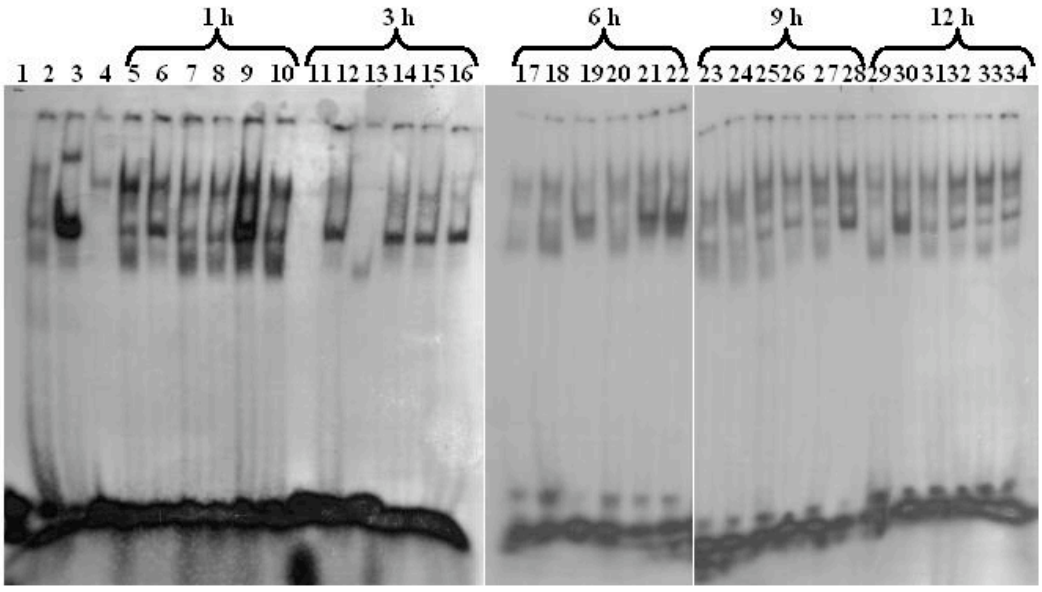


Fig. 7 Binding reactions involving nuclear extracts from untreated and various treatment groups were performed in a total volume of 20 μL containing nuclear extract (with equal amount of protein), 1 μg of poly (dI.dC)-poly(dI.dC), 5% sucrose and P^{32}-labeled oligonucleotide double-stranded probe (50,000 to 100,000 cpm) in 1X binding buffer (Clonetech). The reaction mixture was incubated for 20 min at room temperature. In supershift experiments, 4 μl of anti-p65 antibody (10 X) was incubated with the binding mixture 40 min prior to the addition of probe. The bound complexes were separated from free probe by non-denaturing polyacrylamide gel electrophoresis. The gel was run at 200 V for 2 h, dried and autoradiographed. Lanes 1-4: probe, competition with cold primer, p65 antibody with 25μg protein and p65 antibody with 10μg protein respectively. Lanes 5-10; 11-16; 17-22; 23-28 and 29-34 are UT, V-18, V-20, 2 Gy, V18+2 Gy and V20+2 Gy for each set at 1, 3, 6, 9 and 12 h post-treatment respectively.

Gy radiation, however reduced the binding of p65 at 1 and 6 h compared to other groups. As shown in Figs. 3 b, 6 and Table 1, VAD-20 has more radiosensitizing effects than VAD-18 in PC-3 cells, which could be due to reduced activity of p65 and enhanced expression of pro-apoptotic proteins with simultaneous reduction in anti-apoptotic protein levels shown in Fig. 6.

Chromatin immuno-precipitation (CHIP) assay: To confirm the results obtained from EMSA, CHIP assay was carried out in PC-3 cells using Bcl_2 primers (Bcl_2 as target of p65) followed by hot PCR. Results were normalized with 28SrDNA as the control. Similar to the results obtained from EMSA, enhanced binding of p65 to its target promoter (Bcl_2) was observed by CHIP analysis only after 6 h of treatment leading to increased transactivation activity.

Task 2 c: Immunofluorescence studies: To confirm the time dependent changes observed in the localization and activity of p65, immunofluorescence studies were carried out for p65 and its binding partner p50 in PC-3 cells (Fig. 8). Results obtained from these localization studies reflected the similar pattern. While, p65 and p50 co-localized in the cytoplasm in the control groups, in all the other groups except 5 Gy radiation (co-localization in the nucleus), time dependent changes were observed. The summary of the localization is provided in the table 4. Most importantly, as observed before, V-20 either alone or in combination with radiation showed co-localization of p65 and p50 in the cytoplasm at 3 and 6 h post-treatment time consistent with the observation with Western analysis, EMSA and CHIP analysis.

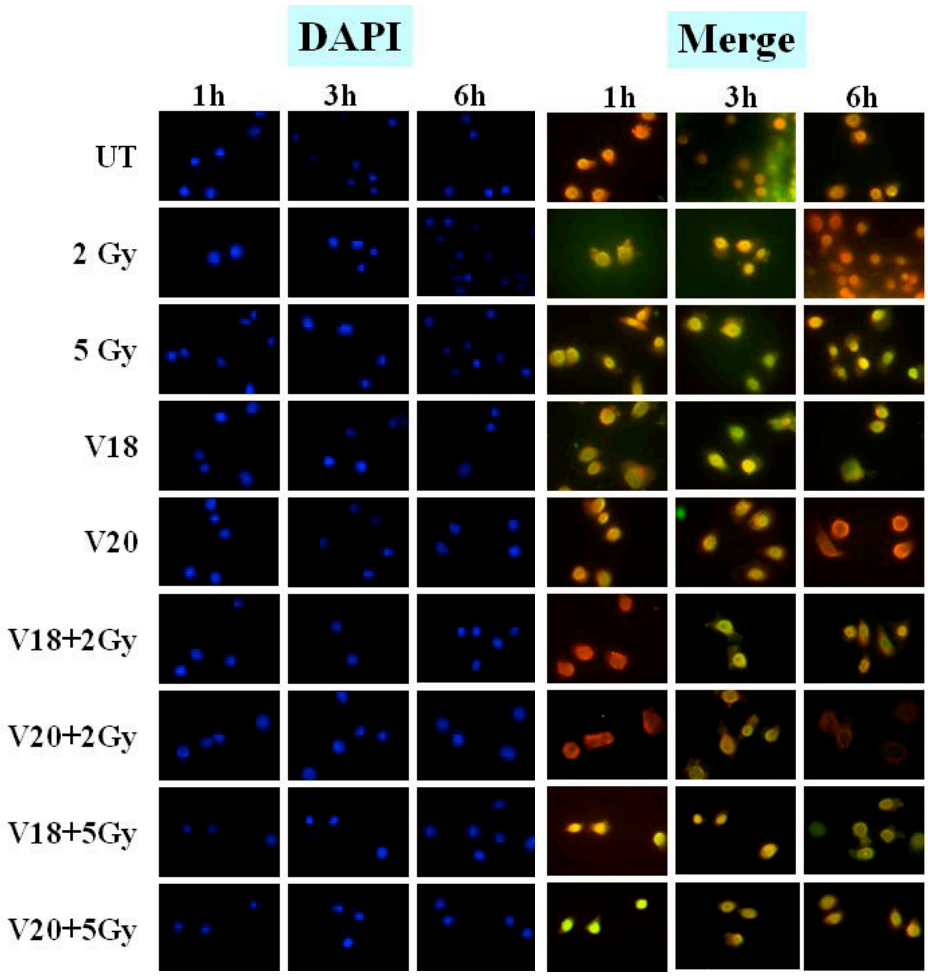


Fig. 8 Effects of VAD-18 (0.5 μM) and VAD-20 (7.5 μM) with or without radiation (2 Gy or 5 Gy) on co-localization of p65 (green) and p50 (red) analyzed by immunofluorescence.

Table 4: Effects of VAD-18 (0.5 μM) and VAD-20 (7.5 μM) with radiation (2 or 5 Gy) on the co-localization of p50 and p65 proteins in PC-3 cells. C-cytoplasm; N-nuclear; PN-perinuclear.

Treatment	UT	2 Gy	5 Gy	V-18	V-20	V-18+2 Gy	V-20+2 Gy	V-18+5 Gy	V-20+5 Gy
1 h	C	N	N	N	C/N	C	C	N	N
3 h	C	N	N	N	C	C/N	PN	N	PN
6 h	C	C	N	C	C	C	C	PN	PN

Since, results obtained from this task showed that pro-survival protein, p65 may be active at certain time points following treatment, there was a need to look for the other transcription factors responsible for increased response in the form of clonogenic inhibition in PC-3 cells. It has been reported before that the duration of the NFκB nuclear activation depends on the activity of HDAC3, which provides an acetylation balance dependent mechanism for the regulation of NFκB-mediated transcription (46). Once acetylated active NFκB dimers are present in the nucleus and they will bind to the chromatin at the newly exposed binding sites. Saccani et al., have shown dual waves of the recruitment of NFκB to target promoters by LPS stimulation in the Raw 264.7 murine macrophage cell line (47). They suggested that a subset of target genes whose promoter is already heavily acetylated (H 4 acetylation) before stimulation, is constitutively and immediately accessible to NFκB and is transcribed immediately after NFκB recruitment, whereas other target genes are not immediately accessible to NFκB. Recruitment of NFκB to late accessible gene promoters occurs after nuclear entry and is preceded by the formation of an initial transcription factor complex that directs the hyperacetylation of the promoter and makes it accessible to NFκB (48). Since, several of the nuclear receptor coactivators, CREB-binding protein (CBP)/adenoviral protein E1A (p300) protein, CBP/p300 associated factor (P/CAF) and ATF-2 all possess intrinsic HAT activity (reviewed in (49)) and are vital for the co-activation of several transcription factors, including NFκB and AP-1 in the transcription machinery (reviewed in (49)), it is likely that HDAC inhibitors by inhibiting the de-acetylation can enhance the formation of such transcription initiation complexes. Therefore, we used the TranSignal protein/DNA array from Panomics that identifies 54 transcription factors in PC-3 cells. The array results are tabulated for the most important factors that showed differences in expression compared to untreated group in table 5.

Following the comparison between the different treatment groups, it was observed that while the transactivation function of the most of the factors involved in the transcription initiation machinery (C/EBP, p65, CREB and AP-1) (reviewed in (49)) or its activators like NFATc (50) were either up-regulated or induced, most of the STATs and upstream stimulatory factor-1 (USF-1) were down-regulated following treatment with HDAC inhibitors alone or in combination with radiation (Table 5).

Although, USFs are shown to be highly versatile stress responsive transcription factors (51), their role in ionizing radiation mediated effects has not been studied till now as per our knowledge. Therefore, it was of great interest to investigate the effects of HDAC inhibitors and radiation on the various targets of this transcription factor.

Table 5: TranSignal protein/DNA array (from Panomics) was carried out according to the manufacturer's instructions in PC-3 cells 24 h after the treatment. Results are presented compared to the controls. "Up" stands for up-regulated and "D" for down-regulated transactivation activity of various transcription factors compared to the untreated group; "In"-Induced when basal levels were not present in the control group and "R"-repressed that is transactivation function was absent in the treated group but was present in the control group. In the groups where significant changes were not observed compared to controls, the cell is left blank.

V18				V20				2 Gy				V18+2 Gy			
Up	**D**	**In**	**R**	**Up**	**D**	**In**	**R**	**Up**	**D**	**In**	**R**	**Up**	**D**	**In**	**R**
		AP-1				AP-1				AP-1				AP-1	
		ARE													
		C/EBP								C/EBP					
CBF				CBF				CBF				CBF			
AP-1 (new)				AP-1 (new)				AP-1 (new)				AP-1 (new)			
		CREB				CREB				CREB				CREB	
		E2F1				E2F1				E2F1				E2F1	
		ERE								ERE				ERE	
		FAST-1								FAST-1					
		GAS/ISRE				GAS/ISRE				GAS/ISRE				GAS/ISRE	
		GATA				GATA				GATA				GATA	
		GRE				GRE				GRE				GRE	
		HNF-4				HNF-4				HNF-4				HNF-4	
				MEF-2				MEF-2				MEF-2			
		Myc-Max				Myc-Max				Myc-Max				Myc-Max	
		NFATc				NFATc				NFATc				NFATc	
		NF-E1				NF-E1				NF-E1					
NFkB				NFkB				NFkB				NFkB			
		Oct-1				Oct-1				Oct-1				Oct-1	
		Sp1								Sp1					
	Stat1			Stat1				Stat1							
	Stat3							Stat3							
	Stat5			Stat5				Stat5				Stat5			
	TR (DR-4)												TR (DR-4)		
	USF-1												USF-1		
	VDR (DR-3)											VDR (DR-3)			
			HSE	HSE				HSE				HSE			

We identified four targets of USF-1 which are involved in cellular proliferation and cell cycle; human telomerase reverse transcriptase (hTERT), IGF2R, Cyclin B1 and Cdk1. Since, USF-1 transactivation function was found to be down-regulated following HDAC inhibitor treatments by protein/DNA array in PC-3 cells, we expected that expression of its target proteins, which are found to be associated with carcinogenesis or proliferation should also be reduced. To confirm this hypothesis, we performed real time RT PCR with the primers from Applied Biosystems. Results are presented in Fig. 9.

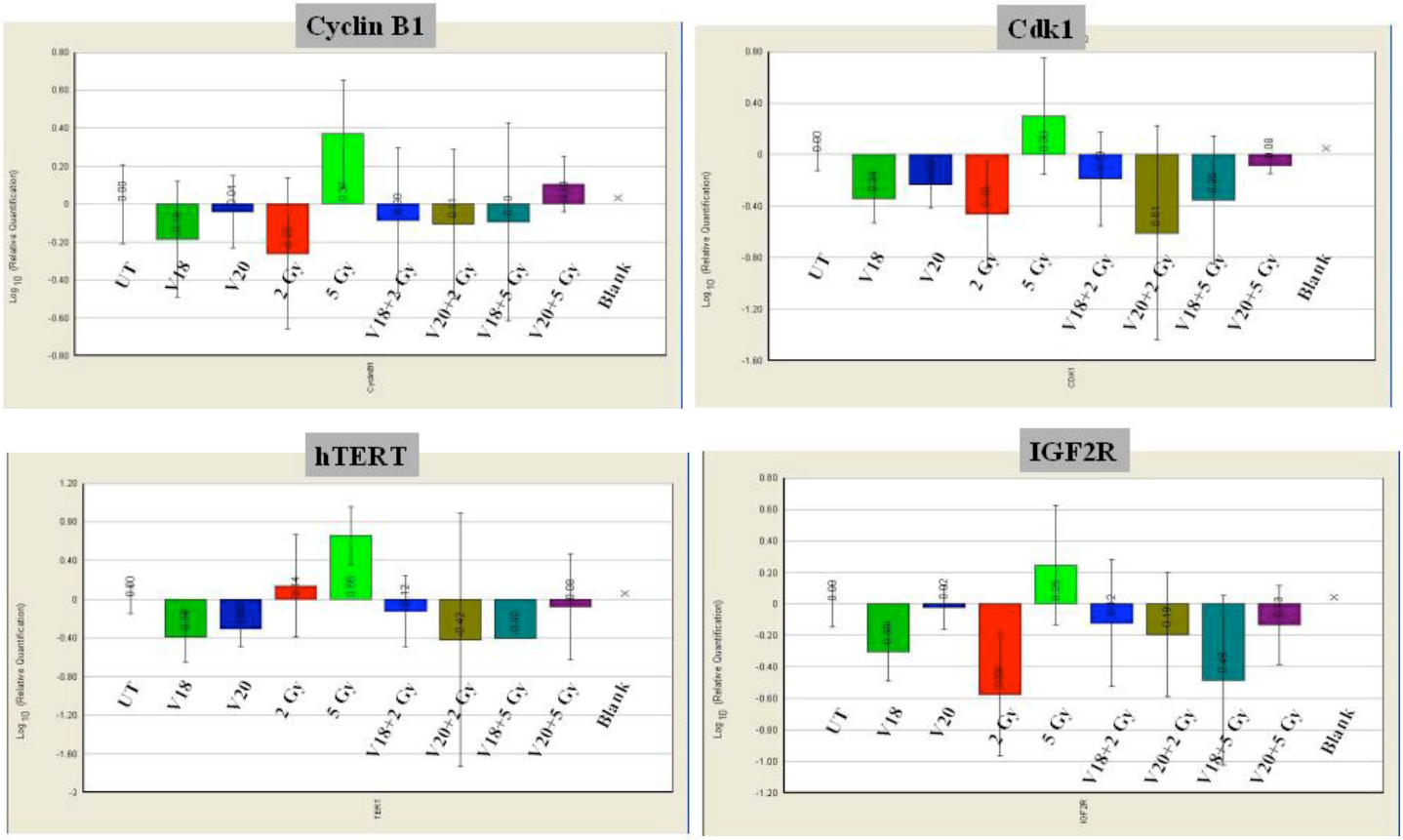


Fig. 9 Following various treatments in PC-3 cells, RNA was extracted and cDNA was prepared using PCR with 1 µg of RNA. Real time PCR was then performed using Applied Biosystems' primers, reagents and instrument. Results are presented after normalization with untreated group and using β actin as the endogenous control.

An induction in gene expression was observed following irradiation of cells with 5 Gy dose for all the four genes. All the other treatment groups however, showed reduction in the expression as expected. Thus, the HDAC inhibitors were able to reduce the expression of radiation-induced genes involved in cell proliferation and cell cycle.

Task 3. To determine the combined effects of HDAC inhibitors plus ionizing radiation on the regression of (i) prostate cancer xenografts (PC-3) in nude mice and (ii) in-situ prostate tumor in TRAMP mice.

a. Implant tumors (PC-3) in nude mice and study the effects of the inhibitors with or without ionizing radiation on tumor growth and animal survival.

b. Study the effects of HDAC inhibitors in combination with ionizing radiation on in-situ tumors in TRAMP mice and follow the regression of the tumor by ultrasound imaging.

To translate the results obtained in cell lines to clinics, it is essential to study the effects of these inhibitors in combination with radiation in tumor bearing animals. We hypothesized that HDAC inhibitors in combination with radiation will inhibit pro-survival gene activity (NFκB and Bcl-2) and upregulate pro-apoptotic events (Bax) and this signaling will enhance the regression of prostate tumor xenografts. In a more basic research setting, the response to treatment can be hard to study because much of the available research material such as cell lines PC-3, LN3 or explants derived from clinical specimens often represent more advanced stage disease. Tumor xenograft model is one of long-standing pre-clinical screening model to set the stage for clinical trials. However, they do not closely mimic *in-situ* tumor situation. *In-situ* tumor model such as "Transgenic Adenocarcinoma of Mouse Prostate (TRAMP)" has been extensively used in understanding the prostate tumor biology. Growth and development of the prostate glands in the TRAMP mice occurs normally until rising circulating levels of androgen direct the prostate specific probasin regulated transgene to express the SV40 early genes (T/t antigens) thereby initiating the transformation process (52). Currently, numerous investigators around the world are using the TRAMP model to study various chemoprevention strategies, including dietary and hormonal manipulation, on the timing, incidence and nature of spontaneous prostate cancer. Furthermore, because the TRAMP mice were generated in the pure C57BL/6 background, they have an intact immune system and thereby facilitate studies designed to exploit the immune response in vaccine based prevention studies, a clear advantage over other "*in vivo*" models that require the use of immunodeficient nude mouse hosts.

Breeding and genotyping: To perform these experiments, we are currently breeding in house TRAMP (transgenic adenocarcinoma mouse prostate) mouse (C57BL/6 x FVB PB-Tag transgene mouse) colony from heterozygous males and females received from Dr Greenberg, Baylor College, Texas. Genotyping of these mice was performed after extracting DNA from the ear punch samples and performing PCR. The cross-breeding data are given in Table 6. We have obtained 16 true TRAMPs till now; 8 male and 8 females. These are being further bred to generate more TRAMP mice.

Table 6. Mouse cross-bread TRAMP population.

Date of Birth	Gender	Genotype	Date of Birth	Gender	Genotype
05/22/05	M	TRAMP+/-	05/14/05	F	TRAMP+/-
	M	TRAMP+/-		F	TRAMP+/-
	M	TRAMP+/+		F	TRAMP+/-
	M	TRAMP+/-		F	TRAMP+/-
	M	TRAMP+/-	05/14/05	M	TRAMP+/-
	M	TRAMP+/+		M	TRAMP+/-
	M	TRAMP+/+		M	TRAMP+/-
	M	TRAMP+/-		M	TRAMP+/-
	F	TRAMP+/-		M	TRAMP+/-
	F	TRAMP+/+		M	TRAMP+/-

	F	TRAMP+/-		M	TRAMP+/-
	F	TRAMP+/-		F	TRAMP+/-
	F	TRAMP+/+		F	TRAMP+/-
	F	TRAMP+/-	05/22/05	F	TRAMP+/-
05/27/05	F	TRAMP+/+		F	TRAMP+/-
	F	TRAMP+/+		F	TRAMP+/-
	F	TRAMP+/-	05/14/05	M	TRAMP+/-
	F	TRAMP+/-		M	TRAMP+/-
03/21/05	M	TRAMP+/-		M	TRAMP+/+
	M	TRAMP+/-		M	TRAMP+/-
02/28/05	M	TRAMP+/-		M	TRAMP+/+
	M	TRAMP+/-	03/27/05	M	TRAMP+/-
	M	TRAMP+/+		M	TRAMP+/-
	M	TRAMP+/-	12/20/04	M	TRAMP+/-
12/20/04	F	TRAMP+/-	05/27/05	F	TRAMP+/-
05/03/05	F	TRAMP+/-		F	TRAMP+/-
	F	TRAMP+/-	05/14/05	M	TRAMP+/-
	F	TRAMP+/-	05/15/05	F	TRAMP+/-
	F	TRAMP+/-		F	TRAMP+/-
05/22/05	F	TRAMP+/-		F	TRAMP+/-
	F	TRAMP+/+	05/14/05	F	TRAMP+/-
	F	TRAMP+/-		F	TRAMP+/+
	F	TRAMP+/-	05/15/05	M	TRAMP+/-
05/27/05	M	TRAMP+/-		M	TRAMP+/-
	M	TRAMP+/+		M	TRAMP+/-
03/27/05	F	TRAMP+/-		M	TRAMP+/-
04/20/05	M	TRAMP+/-	04/20/05	F	TRAMP+/+
	M	TRAMP+/-		F	TRAMP+/+
	M	TRAMP+/-		F	TRAMP+/-
10/17/05	M	TRAMP+/-		F	TRAMP+/-
	M	TRAMP+/-	03/17/05	F	TRAMP+/-
05/14/05	M	TRAMP+/-		F	TRAMP+/-
	M	TRAMP+/-		F	TRAMP+/-
	M	TRAMP+/-	03/21/05	F	TRAMP+/-
	M	TRAMP+/+		F	TRAMP+/-
03/17/05	M	TRAMP+/-	03/17/05	M	TRAMP+/-
				M	TRAMP+/-

Further, to monitor in-situ tumor regression and tumor growth delay after treatment with HDAC inhibitors and ionizing radiation in TRAMP mice, we developed a magnetic resonance imaging technique to acquire prostate images in University of Kentucky. Since, Weis Center for Research (where we have moved) does not have MRI facility, we plan to do the imaging using ultrasound that is available in the animal facility of the Center with the help of urologists. These prostate images will be used for volumetric measurements and treatment planning for radiation.

Ultrasound images will be acquired on day 0 when tumors reach tumor volume of ~5 mm^3, day 5, day 10, day 15, day 20, day 25 and day 30. Volumes will be calculated from these images and regression will be analyzed. The following method will be used for imaging:

Ultrasound imaging methods: Transabdominal ultrasound using 15.0 MHz UltraBand linear transducer (Agilent SONOS 5500, Andover, Massachusetts) will be performed in TRAMP mice. Before ultrasound, the mice will be anesthetized with isoflurane (3% in room air) and the abdomen will be shaved. The volume of the tumor will be estimated using the following formula: length x width x depth x 0.5. This measure has been shown to correlate well with actual tumor size in mice (53). Additional images will be acquired to characterize the tumor's progression. Contours of the identified tumors/prostate will be identified. Volume information will then be extracted utilizing the Dose Volume Histogram (DVH). Temporal tumor volume studies will be achieved by imaging sessions that precede each irradiation time.

For irradiation of tumors in either xenografts or TRAMP mice we proposed to use Cs-137 gamma irradiator. In Weis Center for Research we have two X-ray superficial units (Bucky X-Ray International, Inc. and Therapax 150T, Oldelft Corporation) located in the animal house itself and we plan to irradiate tumors with X-rays rather than gamma rays as proposed in specific aim 3. This should not affect the aim as X-rays are more close to clinical situation where they are used for treatment more frequently than gamma rays.

Administration of radiation therapy: The tumors will be irradiated locally at room temperature by a X-ray superficial unit. The current dose rate is 3.0 Gy/min at the center of the tumor. A uniform dose (± 2%) can be obtained in the center of an 8 cm length. For in-situ TRAMP tumors, 0.5 cm cons will be placed on the lower abdominal area to irradiate the in-situ prostate tumor by specific target alignment. Determination of tumor growth and survival will be made. Statistical significance of differences in survival will be assessed by the method of Kaplan-Meier.

III. KEY RESEARCH ACCOMPLISHMENTS

- VAD-18, VAD-20 and (S)-HDAC-42 are novel HDAC inhibitors that require low doses to modulate hyper-acetylation mediated effects.
- HDAC inhibitors are potential radio-sensitizers *in vitro.*
- Effects of these inhibitors are mediated through cell cycle arrest, down-regulation of anti-apoptotic proteins, upregulation of pro-apoptotic proteins and abrogation of radiation-induced nuclear translocation of p65, thereby, enhancing cell death.
- A novel signaling pathway is identified in which the effects of HDAC inhibitors are mediated through USF-1 transcription factor. Since, till date role of USF-1 in IR-induced effects are not identified, it will be of great importance to dissect this pathway completely.
- Established EMSA protocol to assess the p65 transactivation function in cells treated with HDAC inhibitors and radiation therapy (method accomplishment).

- MRI based imaging of mouse prostate was established (Imaging Protocol accomplishment). However, in Weis Center of Research, ultrasound imaging will be used instead.
- Established external beam radiation treatment planning protocol to irradiate mouse prostate tumors. In Weis Center for Research, superficial X-ray unit will be used to irradiate tumors.

IV. REPORTABLE OUTCOMES

Since the granting period, we have presented the results in three conferences and we are in progress for completing a manuscript for publication. These reportable outcomes were pertaining to the findings of specific aims 1 and 2. The details of the presentations and manuscript are given below (Abstracts are attached in the appendix):

Presentations

1. **Oral Presentation: Seema Gupta**, Ching-Shih Chen and Mansoor M. Ahmed. Radiosensitization of prostate cancer cells (PC-3) by novel histone deacetylase inhibitors. 51st Annual Meeting of the Radiation Research Society, St. Louis, Missouri, April 24-27, 2004.
2. **Invited talk: Seema Gupta**, Ching-Shih Chen and Mansoor M. Ahmed. Histone deacetyalse inhibitors as radiosensitizers in the treatment of prostate cancer. International Conference on Recent Trends in Radiation Biology & 7th Biennial Meeting of Indian Society for Radiation Biology, Mumbai, India, December 1-3, 2004.
3. **Poster presentation: Seema Gupta**, Swati Girdhani, Sara J. Boyer, Ching-Shih Chen, Mansoor M. Ahmed. Novel histone deacetylase inhibitors as radiosensitizers in the treatment of prostate cancer. 97th Annual Meeting of AACR, Washington, DC, USA, April 1-5, 2006.

Manuscript in preparation

- Seema Gupta, Ching-Shih Chen, Mansoor M. Ahmed. Radiosensitization of human prostate cancer cell lines by novel histone deacetylase inhibitors.

V. CONCLUSIONS

The major conclusion of specific aims 1 and 2 demonstrated that HDAC inhibitors can radiosensitize tumor cells. Studies in PC-3 cells demonstrate that these effects are mediated through cell cycle distribution changes, abrogation of translocation of p65 to the nucleus and inhibition of its binding activity. Identification of USF-1 transcription factor by protein/DNA array and real time RT PCR has opened a new avenue for dissecting the signaling pathways induced by HDAC inhibitors leading to radiosensitization effects. However, mechanisms of radio-sensitization and differential effects in normal cells remain to be elucidated completely,

which will help in synthesizing better inhibitors for improving the radiotherapy of cancer. Studies are in progress:

- To understand the role of USF-1 in the radiosensitizing effects of novel HDAC inhibitors: expression, binding, activity, translocation etc as well as by intervention studies.
- To investigate radiosensitizing effects of second generation HDAC inhibitor, (S)-HDAC-42 and to compare the results with SAHA in CWR22RV1 and LnCAP cells.
- To study the radiosensitizing effects of these inhibitors in prostate cancer bearing nude mice and TRAMP mice.

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VII. APPENDIX

Abstract for oral presentation in 51st Annual Meeting of the Radiation Research Society, St. Louis, Missouri, April 24-27, 2004.

Radiosensitization of prostate cancer cells (PC-3) by novel histone deacetylase inhibitors

Seema Gupta, Ching-Shih Chen and Mansoor M. Ahmed

Inhibition of histone deacetylases (HDACs) leads to hyperacetylation of histones which in turn regulates transcriptional activation of specific genes through the relaxation of chromatin conformation. HDAC inhibitors can be used in combination with certain established anti-tumor agents to augment clinical efficacy and/or to reduce toxicity. Since, the studies with these inhibitors in combination with radiation are lacking, the purpose of the present study was to investigate the effects of two novel HDAC inhibitors, VAD-18 and VAD-20 (Zn^{2+}-chelating, short chain fatty acids) in prostate cancer cell line, PC-3 with ionizing radiation (IR). Colony forming assays showed significant radiosensitizing effects with both the inhibitors at IC_{50} concentrations in PC-3 cells (SF_2=0.2±0.013; D_0=145 cGy for VAD-18 + IR and SF_2=0.097±0.02; D_0=122 cGy for VAD-20 + IR) compared to IR alone (SF_2=0.48±0.065; D_0=180 cGy). A significant transient block in G_2/M phase of the cell cycle was observed with either 2 Gy of radiation or drug alone up to 12 h post-treatment. Exposure to VAD-18 resulted in an additional delay following irradiation, which was significant, while VAD-20 had a lesser effect. However, at later time points (after 24-72 h), the block was released. Since, the hypodiploid peak was apparent only at 36 h and later, it appears that only delayed apoptosis was enhanced in combined treatment as cells blocked in G_2/M harbor more damage, which could lead either into apoptosis at a later time or manifest enhanced cytogenetic damage after the release of G_2/M block and lead to secondary apoptosis. Indeed, analysis of cells by fluorescence microscopy showed enhanced cytogenetic damage with radiation and the combination. While, p65 protein (component of NFκB, a survival protein) was present in the nuclear extract till 6 h post-treatment with either drugs or IR alone (Western blot), it disappeared following the combined treatment implying that the inhibitors sensitize the cells to IR by reducing the levels of IR-induced p65 in the nucleus. Further studies to understand the role of cell cycle disturbances and mechanisms by which levels of active NFκB are altered and leads to radiosensitization by these inhibitors are under progress in PC-3 and other prostate cancer cell lines.

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Histone deacetylase inhibitors as radiosensitizers in the treatment of prostate cancer

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Transcriptional control of gene expression is intimately linked to the post-translational modification of chromatin by acetylation, methylation or phosphorylation. Further, acetylation status of the chromatin has been shown to profoundly influence the initiation or progression of cancer. Acetylation status of the chromatin, modulated by histone acetylases (HATs) and histone deacetylases (HDACs), is responsible for chromatin remodeling that is required for gene expression. HATs acetylate histones at the lysine residues thus neutralizing the charge. The resulting relaxation of the nucleosomal core particle leads to transcriptional activation. HDACs on the other hand, remove acetyl groups from acetylated histones leading to chromatin compaction and transcriptional repression. HDACs have been shown to target not only histones but also several other transcription factors like RB, p53, NFκB, ATM and MEF2 for deacetylation.

Since, aberrant activity of HDACs leads to the transcriptional repression of tumor suppressor genes contributing to tumor formation, targeting HDACs with inhibitors would not only disrupt normal transcriptional regulation of specific genes through the relaxation of chromatin conformation but can also be used as a target for improving cancer therapy. Indeed, anti-tumor activities of several HDAC inhibitors have been demonstrated in both tumor cells and xenografted models. One of the most important problems in prostate cancer research is the need to develop an effective treatment for radiation resistant tumors. Induction of pro-survival factors by radiation in prostate cancer has been implicated as one of the factors responsible for resistance to therapy. These radiation-induced pro-survival factors may provide anti-apoptotic signal to evade from cell killing effects of radiation. It may be possible to inhibit the functions of radiation-induced pro-survival factors and enhance radiation-induced apoptosis by the use of HDAC inhibitors to augment clinical efficacy and/or to reduce toxicity. The HDAC inhibitors- phenyl butyrate, sodium butyrate, trichostatin A, SAHA, M344, depsipeptide and a benzamide MS-275 have been shown to enhance radio-sensitivity of some colon, glioma and prostate cancer cell lines. However, their efficacy has been limited by low antiproliferative activity, rapid metabolism and non-

specific mode of action. In addition, the mechanisms of radio-sensitization by these inhibitors have not been elucidated.

Based on the X-ray crystallographic structure of HDAC enzyme, Zn^{2+}-chelating, motif-tethered, short chain fatty acids were developed as novel class of HDAC inhibitors. We have used two of these inhibitors (VAD-18 and VAD-20) having phenylacetic acid and butyric acid respectively as the lead compounds. These novel HDAC inhibitors were potent radio-sensitizers of human prostate cancer cells (PC-3). Effects of these inhibitors were mediated through cell cycle arrest, down-regulation of anti-apoptotic proteins (Bcl_2 and Bcl_{XL}), up regulation of pro-apoptotic protein (Bax) and abrogation of radiation-induced nuclear translocation of p65. These novel inhibitors have been shown to hyperacetylate histones H-3 and H-4 in DU-145 prostate cancer cells in a dose dependent manner and at much lower doses as compared to the parent molecule phenylbutyrate indicating that they are potent HDAC inhibitors.

Further studies understanding the role of cell cycle disturbances and mechanisms by which levels of active NFκB are altered, leading to radio-sensitization by these inhibitors in other prostate cancer cell lines as well as investigations *in vivo* would help in developing alternative effective therapies for the treatment of prostate cancer.

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Abstract #5310; **Novel histone deacetylase inhibitors as radiosensitizers in the treatment of prostate cancer.**

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Inhibition of histone deacetylases (HDACs) leads to hyperacetylation of histones, which in turn regulates transcriptional activation of specific genes through the relaxation of chromatin conformation. Since, the studies with these inhibitors in combination with radiation are lacking, the purpose of the present study was to investigate the effects of three novel HDAC inhibitors: VAD-18 and VAD-20 (Zn^{2+}-chelating, short chain fatty acids) and (*S*)-11 ((S)-HDAC-42), a second generation inhibitor (an optically active α-branched phenylbutyryl derivative) in prostate cancer cell lines, PC-3, DU145 and LN-3 with ionizing radiation (IR). A decrease in surviving fraction in PC-3 cells with increasing concentration of VAD-18 and VAD-20 was observed. VAD-18 (IC_{50}=0.5 μM) was more cytotoxic than VAD-20 (IC_{50}=7.5 μM). Colony forming assays showed significant radiosensitizing effects with both the inhibitors in PC-3 cells compared to IR alone. IC_{50} concentrations of (S)-HDAC-42 and SAHA showed that LN-3 cells were most sensitive to both (S)-HDAC-42 and SAHA compared to DU-145 and PC-3 cells. (S)-HDAC-42 (RER=9.8) was able to enhance the radiation effects in PC-3 cells much more effectively than VAD-18 (RER=1.5) and VAD-20 (RER=2.4). A significant transient block in G_2/M phase of the cell cycle was observed with either 2 Gy of radiation or drugs alone up to 12 h post-treatment by flow cytometry. Exposure to VAD-18 resulted in an additional delay following irradiation, which was significant, while VAD-20 had a lesser effect. An increase in NFκB activity was observed with either the drugs alone or radiation alone as assessed by EMSA. While, p65 protein was present in the nuclear extract till 6 h post-treatment with either drugs or IR alone (Western blot), it disappeared following the combined treatment implying that the inhibitors sensitize the cells to IR by reducing the levels of IR-induced p65 translocation in the nucleus. In addition, levels of Bcl_{XL} and Bcl_2 reduced following combined treatment while levels of pro-apoptotic protein, Bax increased. VAD-20 in combination with 2 Gy, however reduced the NFκB activity. VAD-20 has more radiosensitizing effects than VAD-18 in PC-3 cells, which could be due to reduced activity of NFκB. These studies demonstrate that radiosensitizing effects of HDAC inhibitors are mediated through cell cycle distribution changes, abrogation of translocation of p65 to the nucleus and inhibition of NFκB activity. Studies are in progress to investigate radiosensitizing effects of (S)-HDAC-42 and SAHA in other prostate cancer cell lines and to further understand the role of NF-κB and IκB in the radiosensitizing effects of HDAC inhibitors. It is hypothesized that radiation in combination with HDAC inhibitors may render effects involving non- epigenetic events such as nucleo-cytoplasmic shuttling. This study was funded by a post-doctoral award from DOD-PCRP.